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EpiTect® ChIP qPCR Assay Handbook

For quantitative real-time PCR analysis of
chromatin immunoprecipitation

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Product Use Limitations

EpiTect ChIP qPCR Assays are intended for molecular biology applications. These products are not intended for the diagnosis, prevention, or treatment of a disease.

All due care and attention should be exercised in the handling of the products. We recommend all users of QIAGEN products to adhere to the NIH guidelines that have been developed for recombinant DNA experiments, or to other applicable guidelines.

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I. Background and Introduction

The end-point polymerase chain reaction (PCR) has been the cornerstone for detecting chromatin immunoprecipitation (ChIP) DNA. However, this method requires post-PCR gel electrophoresis, photography, and manual image quantification while acquiring only semi-quantitative binding enrichment results. Real-time PCR (qPCR) is easier to use and provides greater sensitivity, a wider dynamic range, and more quantitative results than end-point PCR. Unfortunately, genomic DNA sequence complexity confounds the design of specific and reliable qPCR assays for ChIP analysis. We have developed specific and efficient ChIP qPCR Assays for promoter activation analyses and other ChIP applications.

Pre-designed EpiTect ChIP qPCR Assays are available to tile all human, mouse and rat promoters in the NCBI RefSeq database from -20 kb to +10 kb relative to their transcriptional start site (TSS). Our average one kb tile interval strikes a balance between resolution and sensitivity for screening individual nuclear or transcription factor binding sites within the tiled promoter region. These tiling assays are especially useful for ChIP-on-chip microarray validation by permitting rapid qPCR measurements for a large number of binding regions. The use of qPCR in these assays also provides a more accurate determination of the non-immune IP background, the input genomic DNA material, and the differential occupancy of the promoter region of interest – all at the same time. See Figure 1 for an overview of the recommended EpiTect ChIP qPCR Assay procedure.

EpiTect ChIP qPCR Assays are specifically designed for SYBR® Green real-time PCR detection. We use an advanced primer design algorithm and experimentally verify each assay to insure amplicon specificity and greater than 90 percent amplification efficiency. To guarantee optimal real-time PCR assay performance, the use of our RT^2 qPCR Mastermixes is required. We have formulated and tested these master mixes together with our ChIP qPCR Assays to provide uniform assay performance under standardized cycling conditions. Each instrument-specific master mix contains SYBR Green and the appropriate reference dye to match the instrumentation available in your laboratory. RT^2 qPCR Mastermixes are available for ABI, Bio-Rad, Stratagene, Eppendorf, Roche and other major real-time PCR instruments.

Benefits of EpiTect ChIP qPCR Assays:

- **Speed and Ease:** Standardized, quantitative, and higher-throughput SYBR Green real-time PCR to measure enrichment at multiple sites simultaneously. Profile more promoter regions at one time in the same ChIP sample preparation.
- **Save Time and Money:** Optimized and ready-to-use assays eliminate assay design trial-and-error allowing more target binding sites to be tested.
- **Guaranteed qPCR Performance:** High amplification efficiency and specificity guaranteed. More accurately quantify all ChIP DNA fractions simultaneously.
- **Comprehensive Coverage:** Tiled assays are available for every human, mouse or rat gene promoter. Facilitates rapid validation of promoter tiling ChIP-on-chip results.

Figure 1: Overview of the EpiTect ChIP qPCR Assay procedure.

II. Materials Provided:

EpiTect ChIP qPCR Assay, forward and reverse primers in one tube, enough for 200 25-µL reactions (10 µM each primer)

Storage Conditions:
EpiTect ChIP qPCR Assays are shipped at ambient temperature but must be stored at - 20 °C upon receipt, where they are guaranteed for 6 months.

III. Additional Materials Required:

A. **EpiTect ChIP OneDay Kit** (Cat. no. 334471)
B. **EpiTect ChIP Antibody Kit** (Cat. no. 334481) OR other ChIP-Grade antibody of choice

C. **RT^2 qPCR Mastermix**
MANDATORY for a Complete and Successful Experiment
Be sure to pick the correct one for the instrumentation in your laboratory.

RT^2 SYBR Green ROX qPCR Mastermix: Specifically designed for:
All ABI and Stratagene Instrumentation
Eppendorf Mastercycler® ep *realplex* Instruments with ROX filter set

Catalog Number	Size
330520	For 200 25-µl reactions (2.5 ml)
330529	For 2000 25-µl reactions (25 ml)

RT^2 SYBR Green Fluor qPCR Mastermix:
Specifically designed for BioRad iCycler®, MyiQ®, and iQ5 Instrumentation

Catalog Number	Size
330510	For 200 25-µl reactions (2.5 ml)
330519	For 2000 25-µl reactions (25 ml)

RT^2 SYBR Green qPCR Mastermix:
Specifically designed for instrumentation that does not require a reference dye:
BioRad (MJ Research) MiniOpticon, Opticon 2, and Chromo 4
Roche LightCycler® 480 System
Eppendorf Mastercycler® ep *realplex* Instruments without ROX filter set

Catalog Number	Size
330500	For 200 25-µl reactions (2.5 ml)
330509	For 2000 25-µl reactions (25 ml)

D. **Equipment:**

1. For recommendations on specific real-time instrumentation (thermal cyclers with fluorescent detection), see the list of master mixes above.
2. Calibrated Micro Pipettor
3. DNase-free / PCR Grade water (H_2O), pipette tips and tubes

IV. Related Products

A. EpiTect ChIP OneDay Kit **Catalog Number 334471**

The EpiTect ChIP OneDay Kit contains sufficient reagents to perform 12 Chromatin Immunoprecipitations and DNA Purifications. The Kit yields high quality ChIP DNA suitable as template for Real-Time PCR analysis

B. EpiTect ChIP Antibody Kit **Catalog Number 334481**

More information regarding the EpiTect ChIP Antibody Kit can be found at (http://www.sabiosciences.com/chipgradeantibody.php).

C. EpiTect ChIP qPCR Arrays **Catalog Number 334211**

More information regarding the EpiTect ChIP qPCR Assays can be found at (http://www.sabiosciences.com/chipqpcrarrays.php**).**

D. EpiTect ChIP qPCR Data Analysis

Free available at: http://www.sabiosciences.com/chippcrarray_data_analysis.php

E. EpiTect ChIP Search Portal

Free available at: http://www.sabiosciences.com/chipqpcrsearch.php

F. RT^2 SYBR Green qPCR Mastermixes

More information regarding the EpiTect ChIP qPCR Assays can be found at (http://www.sabiosciences.com/chipqpcrmastermix.php)

V. ChIP qPCR Assay Protocol:

Please read through this entire protocol before beginning your experiment.

NOTE: Preparing a Workspace Free of DNA Contamination
For accurate and reproducible PCR results, it is very important to avoid contamination of the assay with foreign DNA. Any DNA contamination will artificially inflate the SYBR Green signal yielding skewed gene expression profiles and false positive signals. The most common sources of DNA contamination are the products of previous experiments spread into the air of your working environment. Please follow the recommendations below on how to set up and maintain a working environment free of DNA contamination.

1. Wear gloves throughout the procedure. Use only fresh PCR-grade reagents (H_2O) and lab ware (tips and tubes).

2. Physically separate the workspaces used for PCR setup and post-PCR processing or non-PCR operations. Decontaminate your PCR workspace and lab ware (pipettor barrels, tube racks, etc.) before each new use with UV light or with 10% bleach.

3. Close all tubes containing PCR products once you are finished adding or removing volumes. Before discarding any lab ware (tips or tubes) containing PCR products or other DNA, treat with 10% bleach.

4. Do not leave lab ware (tubes and tip boxes) exposed to the air for long periods of time.

5. Do not open any previously run PCR tubes. Opening tubes after the run can release PCR product DNA into the air where it will contaminate and confound the results of future real-time PCR experiments.

A. ChIP DNA Sample Preparation Considerations

For ChIP preparations and quality control follow instructions specify in the user manual included in the EpiTect ChIP OneDay Kit and EpiTect ChIP Antibody Kits using the primer set provided in the Antibody kit. If you follow your own protocol and/or use another antibody of interest, you might find the following considerations useful.

NOTE: *This section does NOT contain a chromatin immunoprecipitation protocol. It is a protocol overview highlighting preparation considerations that affect ChIP qPCR Assay performance.*

1. **Biological Experiment**

Use at least one or two million mammalian cells per biological experimental condition or replicate *for each* IP fraction. Use the same amount of material for all IP fractions.

NOTE: *The amount and volume recommendations made here are suggestions. The actual amounts of ChIP DNA required to produce high quality, quantitative results must be determined empirically for each experiment. Once determined, this amount should be applied uniformly for all samples in an experiment.*

2. **Cross-Linking and Chromatin Preparation:**

Follow your previously used methodology or the recommendations in your ChIP kit.

3. **Chromatin DNA Fragmentation by Sonication:**

For the best possible balance among resolution, analytical sensitivity and IP efficiency, fragment the chromatin DNA to an average apparent size of 500 to 1500 bp as judged by agarose gel electrophoresis (Figure 2) or Agilent Bioanalyzer DNA NanoChip® based on the characterization of a ***purified*** input DNA aliquot with its cross-linking has been reversed.

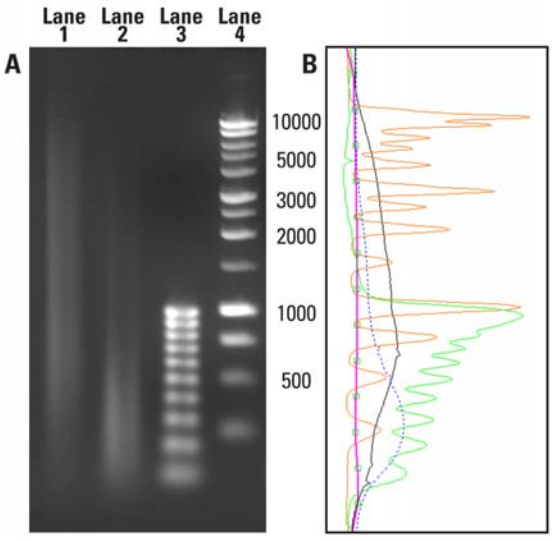

Figure 2: Chromatin Fragmentation Characterization. A) Ethidium bromide stained agarose gel of DNA fragmented by sonication. Lane 1 shows a good fragmentation size range (~800 bp average) for ChIP qPCR Assays, while lane 2 shows a fragmentation size range (~250 bp average) that reduces the measurable signal intensity for ChIP qPCR

tiling assays. Lanes 3 and 4 are 100- and 1000-bp DNA ladder size markers. B) Intensity tracing of the gel lanes showing the range of a DNA fragment sizes superimposed on the size ladders. Lane 1 is the solid line, lane 2 is the dashed line, lane 3 (100 bp ladder) and lane 4 (1.0 kb ladder) are easily identified by comparison to the gel image.

NOTE: *Chromatin fragmentation protocols recommended for ChIP-on-chip tiling microarray analyses yield DNA shorter (~400 to 600 bp average) than the optimal size for ChIP qPCR Assays (~ 500 to 1500 bp average). DNA fragments need to accommodate the longer amplicon size for qPCR (50 to 150 bp) rather than the shorter "probe" size of ChIP-on-chip microarrays (25 to 70 bp). Longer fragment sizes also improve signal intensity but should not exceed 10 kb.*

4. Immunoprecipitation (multiple fractions per sample):

a. Input Genomic DNA – Positive control fraction for data normalization
The Input DNA fraction provides a measurement of the total number of target binding sites before immunoprecipitation. We recommend that one percent of the chromatin sample should be used from each biological experimental condition or replicate. The Input DNA fraction is NOT immunoprecipitated but reserved for processing with the IP fractions in step 5.

b. Divide the chromatin preparation accurately and evenly into at least three equal parts or fractions for the following immunoprecipitations (See Figure 1). Additional fractions may be added to accommodate additional nuclear or transcription factor targets.

Fraction #1 = Non-Immune Serum or IgG (NIS/mock ChIP):
This fraction is the negative control for the non-specific pull-down of DNA during immunoprecipitation, and provides a measurement of the background or noise for the ChIP system.

Fraction #2 = Anti-RNA Polymerase II Antibody (RNA Pol II ChIP):
This fraction is a positive control for successful ChIP when used with a Positive Control Proximal HKG Promoter Assay. This fraction can also provide a measurement of transcriptional activity at a promoter of interest when used with other proximal promoter ChIP qPCR Assays.

Fraction #3 = Anti-Nuclear or Transcription Factor of Interest Antibody (Target TF ChIP):
This fraction is the experimental inquiry that measures the number of specific target sites bound or associated with the nuclear or transcription factor *in vivo*.

Follow your previously used methodology or the recommendations in your ChIP kit for the optimization of immunoprecipitation for each antibody separately.

5. Reverse Cross-Linking:

Follow your previously used methodology or the recommendations in your ChIP kit.

6. DNA Purification:

Follow your previously used methodology or the recommendations in your ChIP kit.

Re-suspend each ChIP DNA fraction in 100 µL of 1 mM Tris, pH 7 to 8 without EDTA. (EDTA inhibits PCR.) This volume recommendation is based on the use of 1 to 2 million mammalian genome equivalents in each IP fraction.

B. Performing EpiTect ChIP qPCR Assays:

NOTE: *The use of RT^2 qPCR Mastermixes is critical for obtaining the most accurate results from EpiTect ChIP qPCR Assays. Be sure to use the correct master mix for your instrument before continuing with this protocol (See Page 6).*

NOTE: *The accuracy and precision of your pipetting determines the consistency of your results. Be sure that all of your micro-pipettors are calibrated before beginning this procedure.*

NOTE: *Be sure that your ChIP DNA meets the quality standards mentioned previously.*

1. **Setup qPCR Assays for Analysis:**

The following is a suggested minimal qPCR Assay setup for quantitative analysis of the experiment and all of the recommended ChIP DNA fractions. There are three (3) levels of variables that must be managed in a ChIP experiment: sample (*e.g.*, treatment or time course), target (IP antibody target), and binding site (specific sequence locations in the genomic DNA).

For **each** sample (experimental condition or replicate), perform each of the following ChIP qPCR Assays defined by their ChIP DNA Fraction (input template) and their qPCR Assay (primer pair):

Table 1: Recommended ChIP qPCR Assays for a Complete Experiment.

Per sample:	ChIP DNA Fraction			
qPCR Assay	Input DNA	Target TF	RNA Pol II	Non-Immune
Queried Site #1	√	√	**X***	√
Negative Control (IGX)	√	√	√	√
Positive Control (Proximal HKG)	√	**X**	√	√

* This qPCR Assay and Fraction combination only provides useful data if RNA Pol II binding to the Queried Site is relevant to the specific experiment.

Therefore, for this experimental example:

(3 Queried Site #1 Assays + 4 Negative Control Assays + 3 Positive Control Assays)
= 10 ChIP qPCR Assays per sample

And, for a minimum of two biological conditions and three replicates each:

(10 qPCR Assays) x (3 biological replicates) x (2 experimental conditions)
= 60 qPCR ChIP qPCR Assays for the complete experiment.

2. Reaction Preparation

For each 25-µL PCR, mix the following components in a PCR tube:

12.5 µL RT^2 qPCR Mastermix, matched with your instrument
6.5 µL ddH_2O
5.0 µL of either undiluted or diluted ChIP DNA template
1.0 µL EpiTect ChIP qPCR Assay primers (10 µM each)
25.0 µL final volume (Final primer concentration is 0.4 µM.)

NOTE: *If improved sensitivity is required, the volume of ChIP DNA template can be increased up to 11.5 µL per reaction IF the DNA solution* **does not** *contain EDTA. Concentration of the ChIP DNA by any means is* **NOT** *recommended, but rather the ChIP preparation should be scaled up or optimized.*

Alternatively:
For improved ease and consistency, ChIP qPCR Assay-specific pre-mixes for each of the three or more DNA regions detected may be prepared. Scale up the following recipe for the number of reactions to be performed within the single experiment and to allow for pipetting error.

Assay-Specific Pre-Mix:
12.5 µL RT^2 qPCR mastermix, matched with your instrument
6.5 µL ddH_2O
1.0 µL ChIP qPCR Assay Primers (10 µM each)
20.0 µL final volume

Then, for each 25-µL PCR, mix the following components in a PCR tube:

20.0 µL Assay-Specific Pre-Mix
5.0 µL of either undiluted or diluted ChIP DNA template
25.0 µL final volume

After all wells have been dispensed, seal the reaction tubes and centrifuge briefly before placing into your real-time PCR instrument.

If more than one PCR plate is required to run all the experimental reactions, dispensed plates may be stored at 4 °C for up to 36 hours or -20°C for 1 week.

3. Performing Real-Time PCR Detection:

NOTE: *The details of programming a thermal cycling profile are instrument-specific, and therefore questions about specific programming functions should be addressed with the instrument manufacturer. Be sure to follow the manufacturer's instructions for the proper operation and maintenance of your real-time instrument. For additional help with instrument setup, see our Instrument-Specific Setup Instructions and Protocol Files at:* ***www.sabiosciences.com/pcrarrayprotocolfiles.php.***

Place your plate, strips, or tubes into the real-time thermal cycler.
Enter and run the following two-step cycling program on your real-time instrument saving the data file with a unique name.

Cycles	Duration	Temperature
1	10 minutes[1]	95 °C
40	15 seconds	95 °C
	1 minute[2]	60 °C

[1] The 10-minute step at 95 °C is required to activate the HotStart DNA polymerase.
[2] Detect and record SYBR® Green fluorescence from every well at the end of the 60 °C annealing / extension step of each cycle.

4. Recommended Quality Control: Dissociation (Melting) Curve

Run your instrument's default melting curve program immediately after the above PCR program, and generate a first derivative dissociation curve. No more than one peak should appear in each reaction at temperatures greater than 75 °C. Record the T_m for each reaction.

If your instrument does not have a default melting curve program, run the following program instead:

95 °C, 1 min; 65 °C, 2 min (OPTICS OFF); 65 °C to 95 °C at 2 °C / min (OPTICS ON).

NOTE: *Be sure to visually inspect the plate after the run for any signs of evaporation from any of the wells. If evaporation is observed, make a note of which wells so that you may qualify your data analysis appropriately.*

C. Data Analysis:

1. Real-Time Data Acquisition and Quality Assessment

NOTE: *The specific application of the recommendations below depends on your real-time PCR instrument software. Please consult the instrument user manual or contact the manufacturer's technical support department for further directions. For consistent results, use the same real-time instrument to generate all results within a single experiment.*

a. Baseline Cycle Range Selection

Allow your real-time PCR instrument's software to automatically define the Baseline Cycles for each individual well. Confirmation of a good range can be performed by viewing the baseline region of the linear-scale amplification curves under high magnification.

b. Threshold Fluorescence Value Selection

Manually position the Threshold level near the mid-point of linear amplification range when inspected in the log-scale view of the amplification curve. The threshold value should be the same for all reactions in the same experiment whether within the same or across separate PCR runs.

c. Real-Time PCR Quality Assessment

i.) Amplification Curve Shape

After cycle number 3 or 4, the Baseline Cycle Range for all reactions should have a constant (flat) level of fluorescence in the Linear View of the amplification curve. In the log-scale view, the slopes, and therefore the efficiencies, of the amplification curves for all the assays should also be parallel to each other to be comparable.

ii.) Dissociation Curve Analysis

Confirm that each reaction produces a single specific product as indicated by a single Dissociation Curve peak at a melting temperature (T_m) greater than 75 °C.

d. Save the qPCR settings as an instrument file (when possible) and export all C_t value data and labels to an Excel spreadsheet.

2. ChIP qPCR Data Analysis ($\Delta\Delta C_t$ method)

NOTE: *ChIP qPCR results can be calculated validly using the $\Delta\Delta C_t$ method[1], because all C_t values for a binding site are determined from the same qPCR assay.*

NOTE: Excel-based ChIP qPCR Data Analysis Template
Download our Excel-based ChIP qPCR Data Analysis template from the SABiosciences web site at the following address:
http://www.sabiosciences.com/manuals/chipqpcranalysis.xls

- Click on the "ChIP qPCR Data Analysis Template" link.
- Save the Excel file to your local computer. Open the file in Excel.
- Follow the instructions for using the template provided in the "Instructions" worksheet.

Our Excel-based ChIP qPCR Data Analysis template provides a guideline and template for performing the following calculations and interpretation of the control reactions.

a. ChIP qPCR Analysis Calculations

Report ChIP qPCR results either as a "% Input" or "Assay Site IP Fold Enrichment" for characterizing individual experimental samples, or as a "Differential Occupancy Fold Change" when comparing multiple experimental samples.

i. Normalize each ChIP DNA fractions' C_t value to the Input DNA fraction C_t value for the same qPCR Assay (ΔC_t) to account for chromatin sample preparation differences.

$$\Delta C_{t\ [\text{normalized ChIP}]} = (C_{t\ [\text{ChIP}]} - (C_{t\ [\text{Input}]} - \text{Log}_2\ (\text{Input Dilution Factor})))$$

Where, Input Dilution Factor = (fraction of the input chromatin saved)$^{-1}$

Average normalized ChIP C_t values for replicate samples.

ii. Calculate the **% Input** for each ChIP fraction (linear conversion of the normalized ChIP ΔC_t).

$$\textbf{\% Input} = 2^{(-\Delta Ct\ [\text{normalized ChIP}])}$$

iii. Adjust the normalized ChIP fraction C_t value for the normalized background (NIS/mock IP) fraction C_t value (first $\Delta\Delta C_t$).

$$\Delta\Delta C_{t\ [\text{ChIP/NIS}]} = \Delta C_{t\ [\text{normalized ChIP}]} - \Delta C_{t\ [\text{normalized NIS/mock}]}$$

iv. Calculate **Assay Site IP Fold Enrichment** above the sample specific background (linear conversion of the first $\Delta\Delta C_t$).

$$\textbf{Fold Enrichment} = 2^{(-\Delta\Delta Ct\ [\text{ChIP/NIS}])}$$

NOTE: *Real-time PCR threshold cycle (C_t) values greater than 36 may not be reproducibly measured and introduce greater degrees of variability into the calculated results. Because the (C_t) values for the background (NIS/mock) ChIP qPCR measurements tend to be in this range, we recommend using % Input values and not Fold Enrichment values to compare experimental samples.*

v. Determine the difference between the normalized experimental sample (S2) and the control sample (S1) ChIP fraction C_t values (second $\Delta\Delta C_t$).

$$\Delta\Delta C_{t\ [S2\text{-}S1]} = \Delta C_{t\ [S2:\text{normalized ChIP}]} - \Delta C_{t\ [S1:\text{normalized ChIP}]}$$

vi. Calculate **Differential Occupancy Fold Change** (linear conversion of the second $\Delta\Delta C_t$ to yield a fold change in site occupancy).

Fold Change in Occupancy = $2^{(-\Delta\Delta Ct\ [S2\text{-}S1])}$

b. ChIP qPCR Analysis Quality Assessment

i. ChIP sample Quality Control
For each ChIP qPCR Assay:

a) The Input DNA Fraction C_t value should be less than 30.

b) The % Input for the NIS/mock IP DNA fraction should be less than 0.01%.

c) The Target TF ChIP DNA fraction C_t value should be at least one cycle less than the NIS/mock IP DNA fraction C_t value (C_t [NIS/mock] – C_t [ChIP] > 1.0) to be considered *quantitatively* above the background signal (noise) for the sample.

ii. ChIP Experiment Quality Control

a) The % Input results from the Positive Control Proximal HKG Promoter Assay for the RNA Pol II DNA fraction should be greater than 0.1%.

b) The % Input of the Negative Control Assays for the Target TF and NIS/mock ChIP DNA fractions should be very similar.

VI. Troubleshooting

A. No Amplification or Very Late C_t Values (> 30) for Input DNA Fractions:

1. Examine each individual amplification curve for evidence of poorly applied or defined baseline and threshold settings.

2. Repeat the qPCR on a limited scale but include a Positive Control qPCR Assay.

   Positive Control qPCR Assay: An assay that uses a genomic DNA preparation from your cell line of interest without cross-linking or sonication at a final concentration of 0.2 ng/μL and that yields a C_t value less than 33

3. If the Positive Control qPCR Assay does not amplify with a C_t value < 33:

   a. Did the instrument perform correctly during the run?

   b. Were all reaction components added to the qPCR pre-mix and properly dispensed into the plate or tubes?

   c. Double check that the qPCR master mix is the correct formulation meant for your real-time instrument, and that it is still in good condition.

      Also see your instrument's troubleshooting guide for additional assistance.

4. If ONLY the Positive Control qPCR Assay amplifies with a C_t value < 33:

   a. The ChIP DNA Fractions are too dilute or too damaged to amplify.

      i. Too little starting material was used for the ChIP preparation.
      Scale up the initial amount of chromatin.

      ii. The chromatin was cross-linked and/or sonicated too much.
      Reduce the cross-linking time and/or the sonication intensity and/or time.

      iii. The DNA isolation procedure has a poor yield or recovery.

      Also see the troubleshooting guide of your ChIP preparation protocol or kit to address these issues in more detail.

   b. Confirm that all reaction components were added to all of the wells.

5. If only one ChIP qPCR Assay primer pair fails to amplify with C_t values < 33 while other assay primer pairs yield more reasonable C_t values:

   a. The ChIP qPCR Assay genomic DNA region may not be intact in the genome used for the experiment. Re-evaluate and re-validate the Positive Control qPCR Assay using un-cross-linked and unfragmented genomic DNA from the same cell line used in the experiment.

NOTE: *Your cell line may contain different sequence relative to the NCBI Genome Build due to unreported chromosomal abnormalities (insertion or deletions) and single nucleotide polymorphisms (SNPs) that affect the ChIP qPCR Assays. First, try selecting the ChIP qPCR Assay in an adjacent tile and repeating the analysis. However, this situation may yet require sequencing of the genome region in your cell line of interest and custom assay development.*

b. The ChIP qPCR Assay primers are not performing to specifications. Contact Technical Support.

B. The Target TF IP DNA Fraction C_t Value is Greater than the NIS/mock DNA Fraction C_t Value:

1. The amount of nuclear or transcription factor binding to the region of interest is at or below the limit of detection (non-specific background) for the ChIP qPCR Assay.

   a. If this phenomenon occurs for some but not all ChIP qPCR Assays, then the result is most likely real and reflects the actual biology of your experimental model system.

   b. If this phenomenon occurs for ALL ChIP qPCR Assays, then the IP of the target nuclear or transcription factor may not be performing efficiently. Reassess and repeat the IP protocol, and re-optimize if necessary.

2. The distance between the ChIP qPCR Assay tile and the target nuclear or transcription factor binding site may be too great for the chromatin fragmentation size range. Insure that your sonication conditions yield an average DNA size of 0.8 to 1.0 kb. Otherwise, look for a more appropriate ChIP qPCR Assay tile or consult or Custom ChIP qPCR Assay design service. Contact Technical Support.

C. No Amplification or Very Late C_t Values for the NIS/mock IP DNA Fractions:

This result is ideal, because it means that the level of non-specific background for this ChIP qPCR Assay is below the limit of qPCR detection. Change the unreported C_t value to 40 and proceed with the recommended calculations of fold enrichment.

D. Low (< 30) C_t Values from the NIS/mock IP DNA Fractions and/or the Negative Control IGX Assays:

The immunoprecipitation is pulling down too much non-specific genomic DNA. Reassess and repeat the IP protocol, and re-optimize if necessary.

E. Positive Control Proximal HKG Promoter Assay and the RNA Pol II DNA Fraction % Input Is Less than 0.1%:

1. The ChIP qPCR Assay genomic DNA region may not be intact in the genome used for the experiment. Re-evaluate and re-validate the Positive Control qPCR Assay as described above.

2. The RNA Pol II IP may not be performing efficiently. Reassess and repeat the IP protocol, and re-optimize if necessary.

Appendix:

1. Detailed Mathematical Explanation of $\Delta\Delta C_t$ Data Analysis Method

Due to the inverse proportional relationship between the threshold cycle (C_t) and the original amount of template DNA, as well as the doubling of the amount of product with every cycle, the original amount of template DNA (L) in each ChIP fraction is expressed as:

$$L = 2^{-C_t}$$

To control for variation between ChIP fractions, the amount of each promoter region of interest in the initial chromatin (Input DNA) fraction provides a convenient normalization factor for the number of genome equivalents used in the analysis. Because the Input DNA fraction only represents one percent of the total material, the Input C_t value is adjusted for this dilution factor by subtracting 6.644 cycles. The ratio of target TF ChIP DNA fraction over the Input DNA fraction determines the fraction of the nuclear or transcription factor sites pulled down in the target TF ChIP DNA fraction.

$$\frac{2^{-C_t(IP)}}{2^{-C_t(Input)}} = 2^{-[C_t(IP) - C_t(Input)]} = 2^{-\Delta C_t} = \text{Fraction of Input}$$

To determine fold-changes in fractional enrichment caused by the experimental conditions and nuclear or transcription factor activation, the normalized amount of the nuclear or transcription factor(s) of interest at the promoter region(s) of interest in the experimental sample is divided by the corresponding normalized amount in the control sample.

$$\frac{2^{-\Delta C_t(expt)}}{2^{-\Delta C_t(control)}} = 2^{-\Delta\Delta C_t} \qquad \text{Where } \Delta\Delta C_t \text{ is equal to } \Delta C_t(expt) - \Delta C_t(control)$$

2. Bibliography

$\Delta\Delta C_t$ Calculations

[1]KJ Livak & TD Schmittgen, Methods, 2001, 25(4):402-8

JS Yuan, A Reed, F Chen & CN Stewart, Jr. BMC Bioinformatics, 2006, 7:85

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[2]SK Chakrabarti, JC James & RG Mirmira, J. Biol. Chem. 2002; 277: 13286 – 13293.

N Rybtsova, E Leimgruber, Q Seguin-Estevez, I Dunand-Sauthier, M Krawczyk & W Reith, Nuc. Acids Res. 2007, 35: 3431 – 3441.

Ordering Information

Product	Contents	Cat. no.
EpiTect ChIP qPCR Assay (200)	Forward and reverse primers (10 µM) for 200 reactions	334001

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit handbook or user manual. QIAGEN kit handbooks and user manuals are available at www.qiagen.com or can be requested from QIAGEN Technical Services or your local distributor.

Notes:

Notes:

Trademarks: QIAGEN®, EpiTect® (QIAGEN Group); iCycler®, MyiQ® (Bio-Rad Laboratories, Inc.); NanoChip® (Caliper Life Sciences); LightCycler® (Roche Group); SYBR® (Molecular Probes, Inc.); Mastercycler® (Eppendorf AG).

Limited License Agreement

Use of this product signifies the agreement of any purchaser or user of the EpiTect ChIP qPCR Assay to the following terms:

1. The EpiTect ChIP qPCR Assay may be used solely in accordance with the *EpiTect ChIP qPCR Assay Handbook* and for use with components contained in the Kit only. QIAGEN grants no license under any of its intellectual property to use or incorporate the enclosed components of this Kit with any components not included within this Kit except as described in the *EpiTect ChIP qPCR Assay Handbook* and additional protocols available at www.qiagen.com.
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